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Pivotal Advance: Pharmacological manipulation of inflammation resolution during spontaneously resolving tissue neutrophilia in the zebrafish

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ABSTRACT

Zebrafish are a unique model for pharmacological manipulation of physiological processes such as inflammation; they are small and permeable to many small molecular compounds, and being transparent, they permit the visualization and quantitation of the inflammatory response by observation of transgenically labeled inflammatory cell populations. Using a transgenic line specifically labeling neutrophils in vivo (mpx:GFP), we studied the effects of a range of pharmacological agents on the resolution of inflammation in vivo. These agents were selected for their ability to modulate neutrophil function and lifespan in human neutrophils in vitro. Agents delaying neutrophil apoptosis (LPS, dbcAMP, and several caspase inhibitors) all lead to a delay in resolution of neutrophilic inflammation. Reciprocally, pyocyanin and roscovitine (inducers of neutrophil apoptosis) lead to reduced neutrophil numbers. The occurrence of apoptosis was observed by time-lapse analysis and confirmed by dual staining for neutrophil-specific mpx activity (TSA staining) and an apoptotic marker (TUNEL). During inflammation, macrophages follow neutrophils into the inflamed site, and TUNEL/TSA dual-positive material can be demonstrated within macrophages, consistent with their uptake of apoptotic neutrophils. This model has several advantages over mammalian models and lends itself to the study of pharmaceutical agents modulating inflammation. *J. Leukoc. Biol.* 87: 203–212; 2010.

Abbreviations: CDK=cyclin-dependent kinase, dbcAMP=dibutyl cAMP, DIC=differential interference contrast, dpf=days postfertilization, eGFP=enhanced GFP, MPO=myeloperoxidase, mpx=myeloid-specific peroxidase, MRC=Medical Research Council, PFA=paraformaldehyde, TSA=tyramide signal amplification, zDEVD.fmk=benzyloxycarbonyl-Asp-Glu-Val-Asp-fluoromethylketone, zETD.fmk=benzyloxycarbonyl-Ile-Glu-Thr-Asp-fluoromethylketone, zVD.fmk=benzyloxycarbonyl-Val-Asp-fluoromethylketone

The online version of this paper, found at www.jleukbio.org, includes supplemental information.

Introduction

Neutrophilic inflammation is a necessary part of the host response against infectious disease. However, neutrophil products are implicated in the tissue damage seen in a range of inflammatory diseases, in a range of organs, including the lung [1]. A therapeutic agent for the removal of neutrophils would be a potential strategy by which inflammatory disease could be treated, and indeed, a precedent for this exists in studies of the CDK inhibitor, roscovitine [2]. One approach to the identification of such an agent is to screen compound libraries for entities with the ability to modulate the inflammatory response. Inflammation is a composite process resulting from dynamic changes in diverse cellular, molecular, and physiological parameters and as such, cannot be modeled adequately in vitro. There is, therefore, a need to identify an in vivo model, in which pharmacological manipulation of neutrophilic inflammation can be readily assessed.

Zebrafish are a vertebrate model organism, which are becoming widely used for the study of the vertebrate immune system [3, 4]. They possess an immune system with many parallels to mammalian systems, including the presence of cells with characteristics of mammalian macrophages, eosinophils, and neutrophils [5–7], and they have been used to study vertebrate inflammation (recently reviewed in ref. [8]).

Using a transgenic zebrafish model of spontaneously resolving tissue neutrophilia [9], we have established a system that permits in vivo tracking of neutrophil fate in real-time. Using bacterial artificial chromosome transgenesis, we have generated a mpx:GFP transgene, which specifically marks zebrafish neutrophils [9]. The myeloid-specific peroxidase (*mpx*) gene is the closest zebrafish (*Danio rerio*) homologue of the mammalian mpo gene

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(*mpo*) [6]. Tail injury by scalpel transection under Tricaine anesthesia leads to recruitment of fluorescent neutrophils, followed by spontaneous resolution of this tissue neutrophilia over the course of 24–48 h, comparable with mammalian models of neutrophilic inflammation [10]. Using this model, we have tested a range of compounds for their ability to modulate the outcome of the inflammatory process. Compounds modulating human neutrophil lifespan *in vivo* were assessed for their ability to modulate inflammation resolution in this model. Compounds tested were caspase inhibitors with differing specificities for a range of caspases, bacterial LPS, and dbcAMP (a stable synthetic cAMP analog). Compounds delaying human neutrophil apoptosis were shown to delay inflammation resolution, and those accelerating neutrophil apoptosis were found to accelerate inflammation resolution. It has proved difficult previously to identify apoptotic neutrophils in the zebrafish model. We show here the identification of apoptotic neutrophils during inflammation resolution and suggest that this process is functionally important in the resolution of inflammation in this model.

MATERIALS AND METHODS

Zebrafish and other reagents

Wild-type (AB strain) or transgenic Tg(*mpx::eGFP*)¹¹¹⁴ zebrafish [9] were maintained according to standard protocols [11]. Reagents were from Sigma (Poole, UK) unless specified otherwise. Rhodamine TUNEL kit (ApopTag® Red) was from Chemicon (Temecula, CA, USA). Rabbit anti-GFP antibody was from Torrey Pines Biolabs (La Jolla, CA, USA), and the polyclonal rabbit anti-zebrafish L-plastin was a kind gift of Paul Martin (University of Bristol, UK). Caspase inhibitor zVD.fmk and zVAD.fmk were from Bachem (Weil am Rhein, Germany).

Compound treatment of zebrafish larvae

Compounds indicated were made up according to the manufacturer's instructions in DMSO, such that the final concentration of DMSO was 1% or less. Appropriate controls of this level of DMSO were included for all experiments. Compound administration was by immersion of the larvae in a solution of the test compound diluted in E3 [11]. For assessment of compound activity in speeding resolution of inflammation, larvae were injured at 3 dpf and recovered. At 4 hours post injury, larvae with a good inflammatory response were arrayed in the test compound in 96-well plates at three fish/well. For practical reasons, plates were incubated at 20°C for 20 h (approximately equivalent to 12 h at 18°C) and then scored by examination under fluorescence stereomicroscopy.

Assessment of neutrophil number

Anesthetized Tg(MPO::eGFP)¹¹¹⁴ larvae were subjected to complete transection of the tailfin using a microdissection scalpel as described previously [9] and recovered for the time interval indicated. The larvae were then re-anesthetized and viewed using a dissecting microscope at 40–100× magnification using a GFP filter set. The number of fluorescent neutrophils at the site of inflammation was counted by eye.

Fluorescein-TSA staining

Larvae fixed in ice-cold 4% PFA were washed in E2 [11] and incubated at 28°C for 10 min in the dark in a 1:50 dilution of fluorescein- or Cy3-tyramide in amplification diluent (TSAplus kit, Fluorescence Systems, Perkin Elmer Inc., Waltham, MA, USA). This was followed by 3 × 10 min washes in E2 and room temperature fixation in 4% PFA for 30 min.

ApopTag Red in situ apoptosis detection staining

Following TSA staining, as described above, samples were washed for 3 × 5 min in PBS before the samples were left at room temperature in 10 µg/ml proteinase K for 90 min. The samples were then washed and stained using the ApopTag Red in situ apoptosis detection kit (Chemicon). The samples were stored in 75% glycerol at –20°C.

Immunofluorescence

For GFP immunofluorescence following TSA staining as above, samples were washed in PBS for 3 × 5 min before incubation with rabbit anti-GFP (1:1000) overnight at 4°C. Secondary incubation was performed using a sheep anti-rabbit Alexa488 secondary at 1:500 for 2 h at room temperature.

Staining for L-plastin immunofluorescence was performed according to the published protocol [12], substituting a sheep anti-rabbit Alexa488 secondary at 1:500 (Torrey Pines Biolabs).

Neutral red staining

Zebrafish larvae were immersed in a 2.5-µg/ml neutral red solution in E2 [5] for 2–4 h [13]. The number of positive cells at the site of injury was counted using an inverted Nikon TE2000 microscope, and a 20× extra long working distance objective. A minimum of five larvae for each data point was assessed.

Image acquisition and processing

Zebrafish larvae were anesthetized by the addition of 0.017% Tricaine to the E3 medium, in which they were maintained [11]. GFP was visualized by excitation at 488 nm. No nonlinear normalization of images was performed. Images were acquired at 20–22°C using an FV1000 Olympus confocal microscope (mounted on a BX61, driven by FV10-ASWv1.4 software) or a Perkin-Elmer UltraVIEW VoX spinning disk confocal microscope mounted on a Zeiss Axiovert 200M, driven by Volocity 4.3 software (Perkin Elmer Inc.). Excitation was with the 488 and 543 laser lines.

Statistical analysis

Data were analyzed (Prism 5.0, GraphPad Software, San Diego, CA, USA) using unpaired, two-tailed *t*-tests for comparisons between two groups and one-way ANOVA (with appropriate post-test adjustment) for other data.

Ethical approvals

These studies have been subjected to appropriate local ethical review and were performed in accordance with UK Home Office legislation. UK law requires that where possible, experiments are performed on animals not protected under the Animals (Scientific Procedures) Act. Therefore, where possible, experiments were performed on unprotected embryos, <5.2 dpf. Older embryos were used where necessary for identification of apoptotic neutrophils, as numbers of neutrophils were higher at this age.

RESULTS

Pharmacological modulation of inflammation resolution in vivo

Previous work in human neutrophils has identified a range of compounds that modulate neutrophil lifespan. Earlier experiments in the zebrafish model had suggested to us that caspase inhibition with a dipeptide pan-caspase inhibitor zVD.fmk [9] was able to delay inflammation resolution. However, whether this relates to altered cytokine processing or altered neutrophil apoptosis is not clear. To explore this phenomenon further, a range of inhibitors of neutrophil apoptosis was tested for their ability to modulate the resolution of inflammation. The aspect of inflammatory process most pertinent to these studies is the

number of neutrophils representing the cellular component of inflammation and is assessed by counting of infiltrating inflammatory cells, marked by the transgene GFP. Previous experiments have defined the time course of inflammation, and 6 h post-injury represents the peak inflammation, and 24 h represents a time-point at which inflammation has largely resolved in untreated larvae. Counting the number of inflammatory neutrophils at two time-points in the same larva permits an analysis of the change in neutrophil number over time in individual animals. This is particularly useful when looking for differences not apparent in pooled batches of wild-type fish, which can only be examined at a single time-point. In control animals, between 6 h and 24 h after injury, the mean (\pm SEM) reduction in neutrophil numbers is $48.1 \pm 7.9\%$ [9]. The most potent caspase inhibitor tested (zVD.fmk, a dipeptide inhibitor of all caspases) reduces this figure to $0.5 \pm 8.1\%$ (Fig. 1A, shown for comparison) [9]. A caspase inhibitor with increased specificity for caspases-3, zDEVD.fmk has the next-largest effect on inflammation resolution (Fig. 1B), which might be expected as a result of the downstream effector nature of this caspase. An inhibitor with increased specificity for caspase-9 also delayed resolution (Fig. 1C). Inhibitors with increased specificity for caspase-8, zIETD.fmk did not show a statistically significant difference in these experiments. This might support a role for mitochondrial pathways of apoptosis in inflammation resolution in this model, and this would be supported by existing data from human neutrophils [14, 15].

Two other agents predicted to delay neutrophil apoptosis in vivo also affect inflammation resolution: bacterial LPS [16–18] and dbcAMP [19] (Fig. 1, E and F). These data suggest that this model is suitable for identifying agents that delay inflammation resolution and further support the hypothesis that neutrophil apoptosis is functionally important in this process.

For the identification of potential therapeutics, however, it is important to test whether the reciprocal situation is also true: whether we can assay for the acceleration of inflammation resolution by removal of inflammatory neutrophils. In vitro agents shown to accelerate neutrophil apoptosis include pyocyanin and roscovitine. Pyocyanin is a phenazine pigment exotoxin of *Pseudomonas aeruginosa*, which is a profound inducer of neutrophil apoptosis in human and mouse models [20, 21]. To test the effects of this agent in the transgenic model of inflammation resolution, we tested pyocyanin initially for its ability to reduce the number of neutrophils recruited to a site of tissue injury. Figure 2A shows neutrophil numbers at a single time-point during the initiation phase of inflammation; hence, absolute neutrophil numbers are shown. The number of neutrophils present at the site of inflammation was reduced by pyocyanin treatment, reflecting a blockade of recruitment or an induction of neutrophil apoptosis. The effect of pyocyanin on neutrophil number was reversed by the addition of zVD.fmk, confirming pyocyanin induces caspase-dependent cell death in this experimental paradigm [22]. Although neutrophil apoptosis induction might be expected to reduce neutrophil numbers, if this were a result of an unphysiological loss of neutrophils, and clearance were overwhelmed, secondary

necrosis would ensue, causing more tissue damage and increased inflammatory cell recruitment. To exclude this possibility, we repeated these experiments to assess the effect of apoptosis induction during prolonged exposure during the resolution phases of inflammation. For these experiments, inflammation was initiated as before and was allowed to progress for 4 h. At that time, larvae with robust inflammatory responses were selected and incubated with media alone, DMSO, pyocyanin, or roscovitine. After a further period in control and DMSO-treated larvae, there were significant numbers of neutrophils persisting at the inflammatory site. Pyocyanin was added at 4 h post-injury at a time when inflammation is established, and the effects on inflammation resolution were assessed at a time when inflammation is resolving, although there are still a significant number of persisting neutrophils in untreated larvae. There were fewer persisting neutrophils in the pyocyanin-treated group, suggesting that pyocyanin has accelerated the resolution of inflammation (Fig. 2B).

Roscovitine, an inhibitor of CDKs, has been shown to be an inducer of neutrophil apoptosis and in addition, is able to induce inflammation resolution in a variety of murine models of inflammation [2]. We therefore tested roscovitine alongside pyocyanin for its ability to induce resolution of inflammation. The number of persisting neutrophils was reduced following roscovitine treatment to a level comparable with that seen with pyocyanin treatment.

A preliminary compound screen to identify compounds accelerating inflammation resolution

The ability to reduce the number of inflammatory cells at a site of tissue injury, as can be seen with pyocyanin and roscovitine in this model, is a potential therapeutic strategy for the treatment of inflammatory disease. We therefore sought to show the potential use of this model for drug discovery, by performing a preliminary, proof-of-principle compound screen to identify compounds accelerating inflammation resolution. The experimental design was as described above, except that for convenience larvae were incubated at 18°C overnight to lengthen the time to reach a suitable end point. From a 960 compound subset of the spectrum collection (MicroSource Discovery, Gaylordsville, CT, USA), we identified 12 compounds with activity in this assay. Of these, six were known to possess anti-inflammatory activity. Of these, flumethasone (a corticosteroid in use in veterinary practice) is the best-characterized and exhibits a convincing dose-response relationship (Fig. 2C). The effects of corticosteroids in the zebrafish inflammation model have been reported previously [23], confirming the validity of this unbiased method for the identification of anti-inflammatory compounds. Flumethasone suppresses numbers of neutrophils at the site of injury below levels seen with pyocyanin, the positive control compound. This confirms the ability of this model to identify anti-inflammatory compounds, and it would be expected that novel compounds might be identified through a similar screen of novel compounds.

Visualization of apoptotic morphology in neutrophils in vivo

Studies by other research groups have failed to identify apoptotic neutrophils in zebrafish inflammation [24, 25]. As

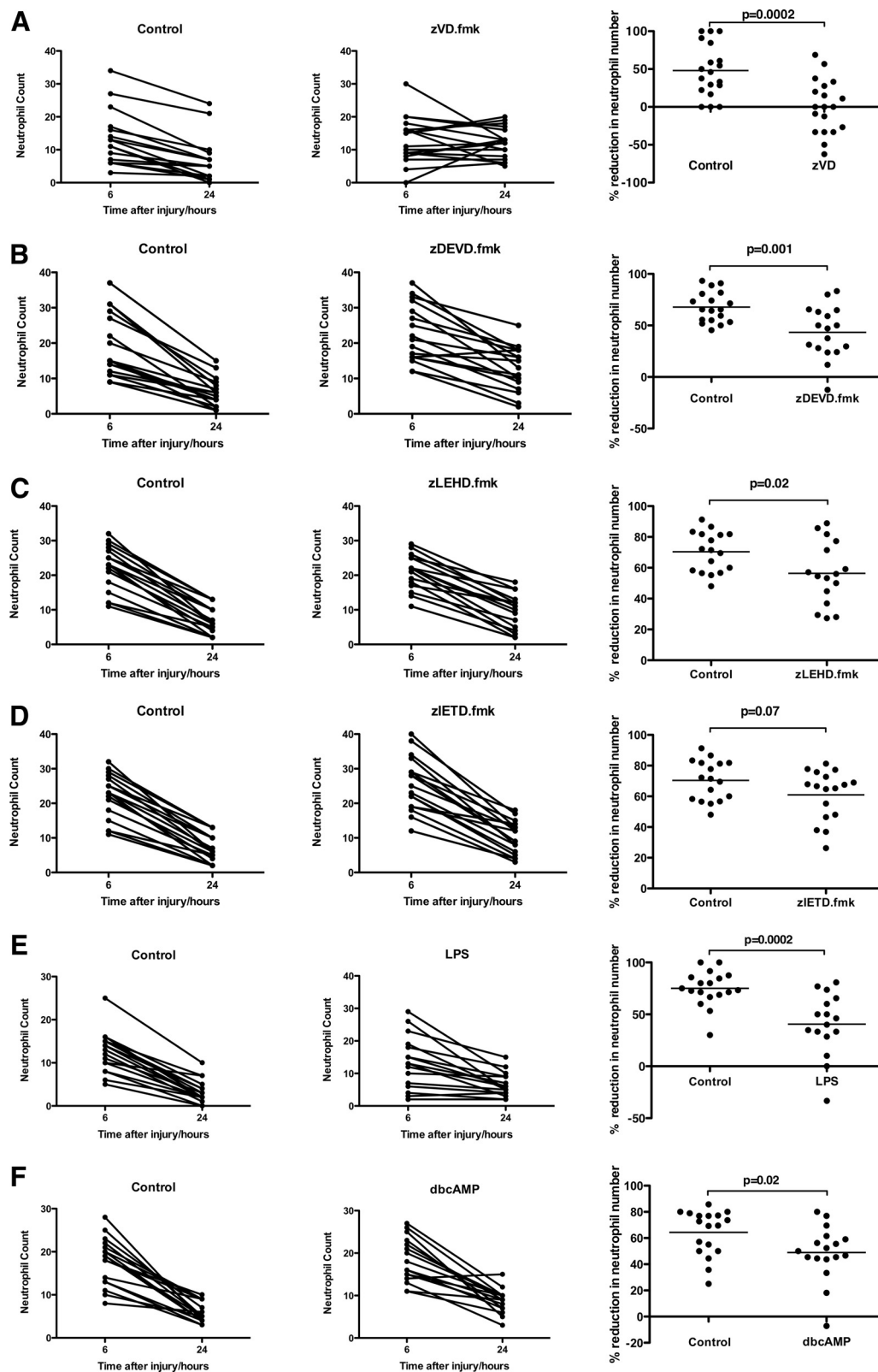


Figure 1. Pharmacological delay of neutrophil apoptosis is associated with a defect in resolution of the cellular component of the inflammatory response following tissue injury in the zebrafish. (A–F) Three dpf mp α :GFP zebrafish were injured by tailfin transection, as described previously [9]. Counts of fluorescent neutrophils at the site of injury were made at 6 h and 24 h after injury. For each fish, the percent reduction in neutrophil numbers between these time-points was calculated. This is a measure of the percentage resolution of the neutrophilic component of the inflammatory response. Each data point corresponds to counts from a single fish. The compounds in question or a comparable vehicle control were added at 4 h after injury. (A) zVD.fmk 100 μ M (pan-caspase inhibitor, Calbiochem, UK); (B) zDEVD.fmk 100 μ M (caspase-3 inhibitor, Bachem, Germany); (C) benzyloxycarbonyl-Leu-Glu-His-Asp-fluoromethylketone (zLEHD.fmk), 100 μ M (caspase-9 inhibitor, Calbiochem); (D) zIETD.fmk 100 μ M (caspase-8 inhibitor, Calbiochem); (E) highly purified bacterial LPS 1 μ M (Alexis Pharmaceuticals, San Diego, CA, USA); (F) dbcAMP 100 μ M (Sigma). *P* values were generated by two-tailed unpaired Student's *t*-test.

the data presented here suggest that neutrophil apoptosis may be functionally important in inflammation resolution, we sought supporting evidence for neutrophil apoptosis in the resolution phase of neutrophilic inflammation.

Following tail transection in mp α :GFP zebrafish and using spinning disk confocal microscopy, we identified neutrophils participating in the inflammatory response. Among these, neutrophils were seen that exhibited morphological features typi-

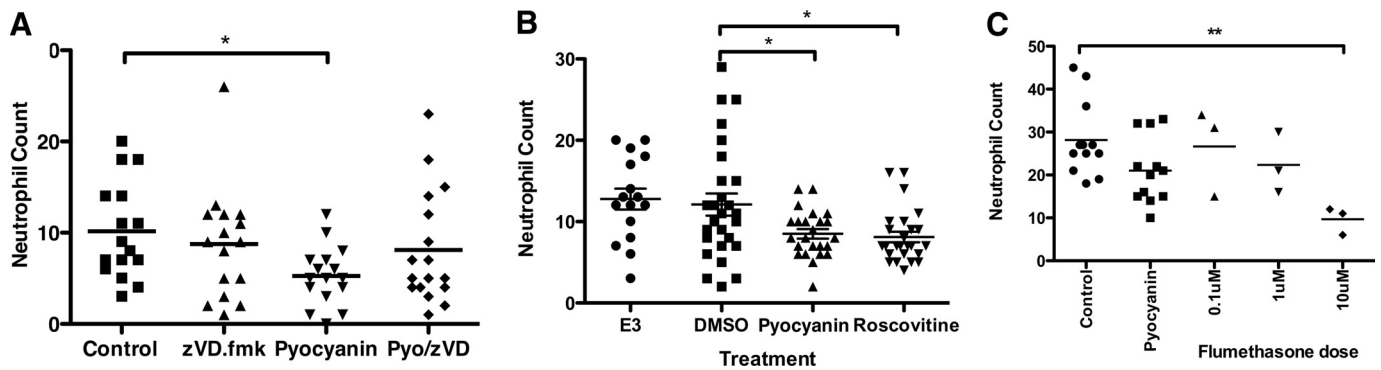


Figure 2. Pharmacological acceleration of neutrophil apoptosis is accompanied by a reduction in neutrophil number at the inflammatory site. Three dpf zebrafish larvae were subjected to tail transection under anesthetic, as described in Materials and Methods. (A) Pyocyanin (Pyo), a phenazine pigment exotoxin of *P. aeruginosa* (50 μ M, a kind gift of Graham Taylor, University College London, UK; purified as described previously [20]), was added at 4 h after injury and counts of neutrophil number made after a further 4 h. The effect of pyocyanin on reducing neutrophil number was blocked by zVD.fmk, a pan-caspase inhibitor (100 μ M). *, $P < 0.05$, for control versus pyocyanin; not significant for control versus other columns, one-way ANOVA with Dunnett's post-test correction; $n =$ at least 16, performed as three independent experiments. (B) Roscovitine, an inhibitor of CDK (20 μ M), DMSO, or pyocyanin (50 μ M) was added 4 h following tailfin transection. After a further 20 h (at 20°C, approximately equivalent to 12 h at 28°C), following addition of the compounds, neutrophil number was assessed by manual counting. Pyocyanin and roscovitine reduce neutrophil number at this time-point. For the comparisons indicated by capped lines, * denotes a P value of <0.05 , ascertained by one-way ANOVA with Dunnett's post-test correction; $n =$ at least 24, performed as three independent experiments. (C) Flumethasone at the dose shown, DMSO (negative control), or pyocyanin (50 μ M, positive control) was added at 4 h following tailfin transection as above. After a further 20 h (at 20°C, approximately equivalent to 12 h at 28°C) following addition of the compounds, neutrophil number was assessed by manual counting. **, $P < 0.01$, ascertained by one-way ANOVA with Dunnett's post-test correction.

cal of apoptosis, namely, loss of locomotion and loss of pseudopod formation and rounding (Fig. 3 and Supplemental Movies 1 and 2). At an interval after the assumption of a rounded shape (66 min in this example), there is an abrupt loss of GFP fluorescence (Fig. 3A and Supplemental Movies 1 and 2). This loss of fluorescence occurs within a single interval between successive frames (i.e., <30 s) and is not accompanied by a loss of the cell corpse, which is seen to persist on accompanying DIC images (Fig. 3B). Loss of GFP is never seen

in cells that have normal morphology or are moving or extending pseudopodia, suggesting that it is specific to the process of apoptosis in these cells.

Biochemical confirmation of neutrophil apoptosis

To confirm these morphological changes do indeed represent neutrophil apoptosis, identification of GFP-positive cells exhibiting biochemical markers of apoptosis would be required. As GFP within apoptotic neutrophils ceases to fluoresce following

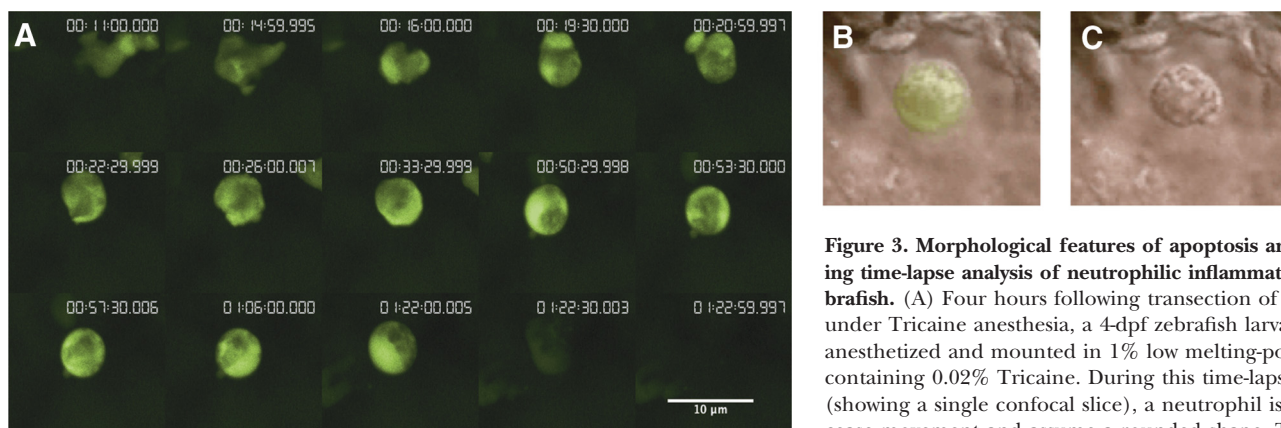


Figure 3. Morphological features of apoptosis are seen during time-lapse analysis of neutrophilic inflammation in zebrafish. (A) Four hours following transection of the tailfin under Tricaine anesthesia, a 4-dpf zebrafish larva was re-anesthetized and mounted in 1% low melting-point agarose containing 0.02% Tricaine. During this time-lapse series (showing a single confocal slice), a neutrophil is seen to cease movement and assume a rounded shape. This is followed by loss of GFP fluorescence. Times since initiation of

time-lapse are shown (h:m:s.ms). The images have been selected to be representative of the series, which can be seen in full in Supplemental Movies 1 and 2. Photomicrographs were taken with a $\times 40$ Plan NeoFluar oil immersion objective, NA1.3 (Zeiss). As a result of the thickness of the tissue section here, the individual cells are not visible on DIC images. Exposure times were minimized, and there was no evidence of any adverse effects over 16 h of imaging. Similar events are seen under wide-field time-lapse microscopy. (B and C) A similar event occurring in the tailfin enables imaging under DIC of the persisting neutrophil cell "corpse." (B) A fluorescent neutrophil showing features of apoptotic morphology in the tailfin during inflammation resolution. (C) The same neutrophil, a single frame (30 s) later, showing that the neutrophil corpse persists, and the GFP signal has been lost completely.

the morphological changes of apoptosis, a second marker of neutrophil lineage was used. It is known that following apoptosis of mammalian neutrophils in tissue culture, endogenous MPO activity persists, and apoptotic neutrophils can be visualized by histochemical peroxidase stains [26]. Therefore, to confirm biochemically that neutrophil apoptosis was occurring, fluorogenic peroxidase substrates (TSA) were used to stain for mpx activity in wild-type fish, fixed at 12 h after injury [25]. TSA-stained larvae were then stained for apoptosis-specific DNA cleavage by TUNEL. In these fish, double-positive cells (positive for neutrophil and apoptosis markers) were observed always to have the typical rounded morphology of apoptotic cells (Fig. 4, A and B). It should be noted that this occurs independently of transgene expression and in vivo fluorescence imaging. The mean number \pm SEM of dual-positive cells seen (at the site of injury) per fish was 3.0 ± 0.6 ($n=8$). Total mean \pm SEM number of neutrophils at this site is 67.8 ± 6.2 . Thus, at this time-point, 4.4% of detectable neutrophils are apoptotic. This is consistent with numbers seen in mammalian models and disease states [27, 28] and is likely to be a contributor to inflammation resolution in this model.

These experiments were repeated using the Tg(mpx:GFP)ⁱ¹¹⁴ line, using TUNEL staining to mark apoptosis and an anti-GFP antibody to mark neutrophils. The number of neutrophils and rates of change of neutrophil number were comparable in the transgenic line and wild-type lines (data not shown). However, there was a dramatic reduction in the number of dual-positive cells seen using this technique. The mean number of apoptotic neutrophils observed in the

Tg(mpx:GFP)ⁱ¹¹⁴ fish was 0.2 ± 0.2 (approximately one for every five fish examined; $P=0.02$ compared with numbers seen with FITC-TSA histochemical mpx staining). An example of apoptotic neutrophils in a larva stained with anti-GFP antibody is shown in Figure 4B and Supplemental Movie 3.

Modulation of rates of apoptosis by inflammatory modulators

To confirm that some of the alterations in rates of inflammation resolution seen above were a result of alterations in rates of apoptosis, we tested a number of compounds for their ability to suppress apoptosis of neutrophils during inflammation resolution in vivo. Zebrafish larvae at 10 dpf were anesthetized and subjected to tailfin transection as above and the number of apoptotic neutrophils at 12 h postinjury assessed by counting of dual (FITC-TSA/rhodamine TUNEL)-positive cells (Fig. 4C). All compounds tested significantly suppressed levels of apoptosis, suggesting that at least part of their action is via a specific delay of neutrophil apoptosis.

Macrophage uptake of apoptotic neutrophils

The low numbers of visible apoptotic neutrophils seen in this and other models of resolving inflammation might be a result of rapid recognition and uptake of apoptotic neutrophils by macrophages [26]. This is necessary, as apoptosis without uptake of the apoptotic corpse permits secondary necrosis to occur, with release of the histotoxic granule contents of the neutrophils. A proportion of the macrophages entering the site of

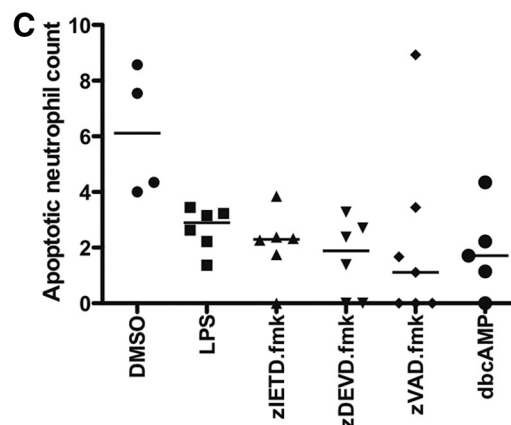
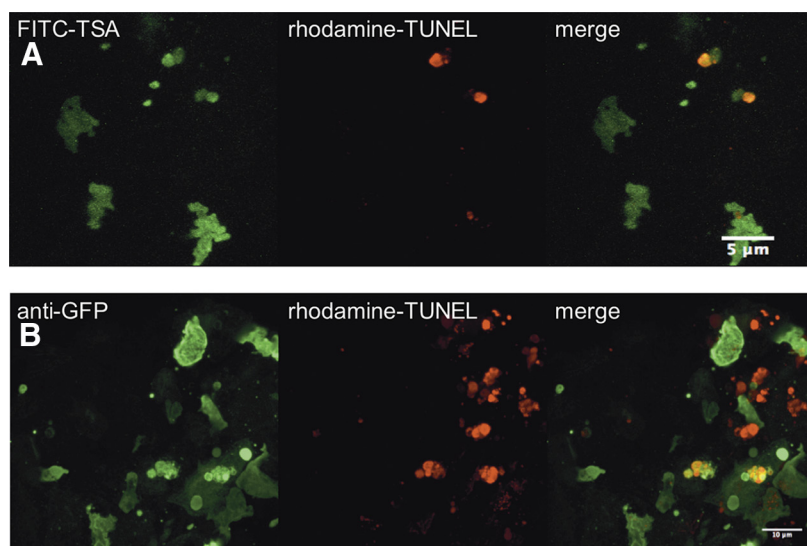


Figure 4. Dual staining for neutrophil-specific mpx and DNA nick-ends (TUNEL) provides biochemical confirmation of neutrophil apoptosis during resolution of neutrophilic inflammation in unmanipulated zebrafish embryos. Seven dpf zebrafish were injured and fixed 12 h later in

4% paraformaldehyde at 4°C. Neutrophils were revealed by (A) FITC-TSA staining (wild-type fish) for peroxidase activity or (B) immunostaining for GFP (mpx:GFP fish), in combination with TUNEL staining with the ApopTag Red kit. Dual-positive cells representing apoptotic neutrophils can be seen in the right panel of A and B. The images shown are maximum-intensity projected images of at least 20 sections in the Z dimension. Images were taken using a $\times 60$ Plan Apo Oil immersion NA1.40 (Olympus). Colocalization of fluorescence in single sections can be seen in Supplemental Movie 3. (C) Larvae at 10 dpf were injured as described previously and treated with the compounds indicated. At 12 h after injury, larvae were fixed and stained for colocalization of neutrophil (FITC-TSA) and apoptotic (TUNEL) markers as above. Dual-positive cells and total neutrophil count were scored from confocal images. Data shown are percent neutrophils costaining with TUNEL markers (i.e., apoptotic neutrophils). $P < 0.05$ for all treatments compared with DMSO control by one-way ANOVA with Dunnett's post-test correction. zVAD.fmk, zVD.fmk.

inflammation in the zebrafish tail-wounding assay can be visualized by staining with neutral red, a vital dye taken up by macrophages by lipid pinocytosis [5]. When macrophages are stained in this way at a variety of time-points in mpx:GFP transgenic larvae, the time course of macrophage and neutrophil migration to an injury site can be observed (Fig. 3A). The peak number of neutral red-positive macrophages follows the peak number of neutrophils by 4–6 h, and these macrophages persist at the inflammatory site, beyond the time when neutrophilic inflammation has resolved.

To demonstrate the remains of ingested neutrophils within macrophages, zebrafish phagocytes were stained by L-plastin immunofluorescence [29] and the presence of mpx identified using Cy3-TSA staining. Two populations of myeloid cells could be distinguished: neutrophils were L-plastin-positive/Cy3-TSA-positive, and macrophages were L-plastin-positive/Cy3-TSA-negative. In uninjured fish, no Cy3-TSA-positive material was seen within macrophages. However, at 12 h postinjury in 7 dpf fish, macrophages containing small, localized foci of Cy3-TSA-positive neutrophil material could be identified clearly. This material was always seen as small circular bodies within the macrophages, consistent with macrophage ingestion of apoptotic neutrophils (Fig. 5, B and C, and Supplemental

Movies 4 and 5). In a further experiment, using triple staining for neutrophilic material (Cy5-TSA), apoptotic markers (rhodamine TUNEL), and leukocyte markers (L-plastin immunohistochemistry), it was possible to identify rare examples of macrophages containing triple-positive, small rounded inclusions, consistent with ingestion of apoptotic neutrophil material (Fig. 5D). This is consistent with a functional role for neutrophil apoptosis and macrophage clearance in the resolution of inflammation in this model.

DISCUSSION

We have identified a range of pharmacological agents that modulate the outcome of experimentally induced inflammation in an in vivo model of spontaneously resolving inflammation. These agents also modulate neutrophil apoptosis in mammalian systems. We have also shown that apoptotic neutrophils can be identified during inflammation resolution in this model at levels consistent with a role in inflammation resolution. The relative contribution of apoptosis and other forms of inflammation resolution, such as retrograde chemotaxis, has not been explicitly investigated.

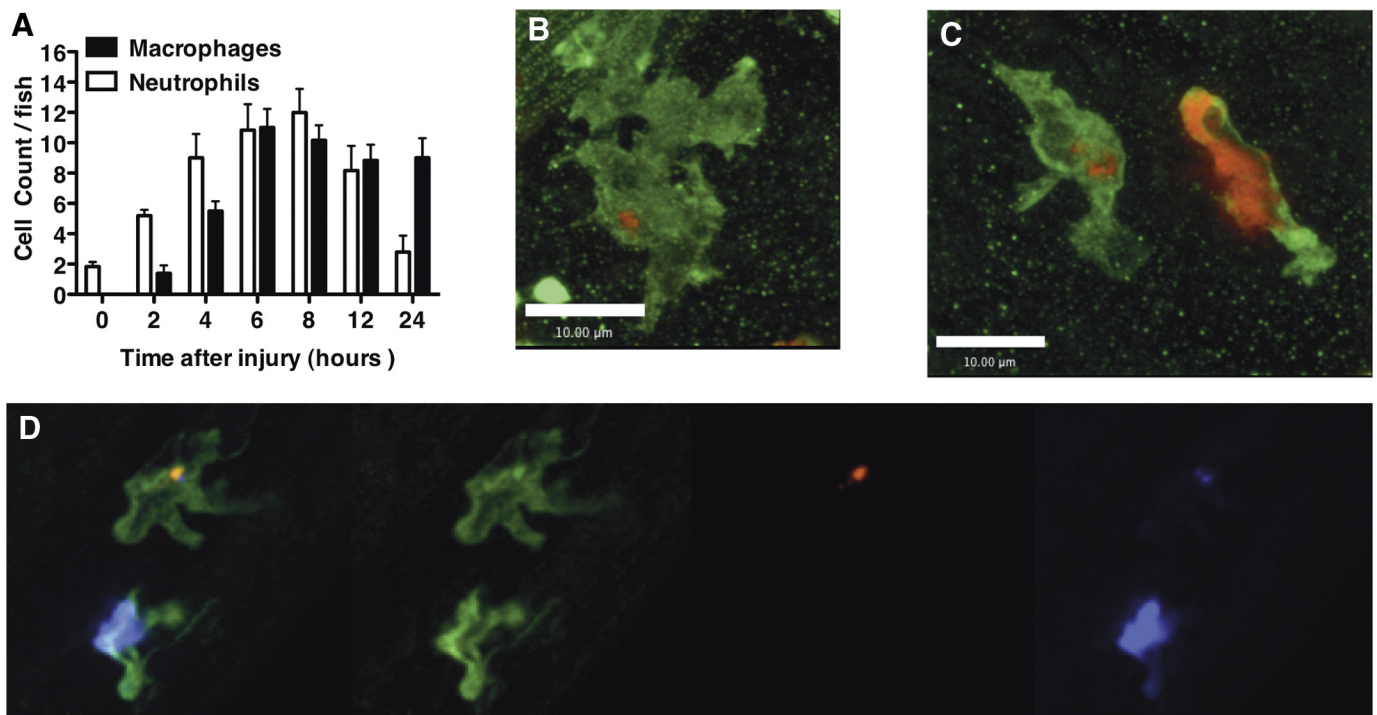


Figure 5. Macrophages take up apoptotic neutrophils during inflammation resolution in vivo. (A) Three dpf mpx:GFP zebrafish were injured as described previously. Two hours before the time indicated, larvae were stained with neutral red for 2 h. The number of neutral red-positive macrophages and GFP-positive neutrophils was counted for each fish under bright-field illumination and 488 nm fluorescent illumination, respectively. (B and C) Seven dpf wild-type zebrafish were injured and fixed 12 h later in 4% paraformaldehyde at 4°C, followed by staining for mpx activity and L-plastin immunohistochemistry, as described in Materials and Methods. Single macrophages can be seen containing TSA-positive neutrophilic material. (C) A neutrophil is shown alongside for comparison. (D) Cy5-TSA staining (purple) was combined with L-plastin staining (green) and TUNEL staining (red) to show colocalization of apoptotic markers and neutrophil markers within a macrophage. Three-dimensional reconstructions are shown in Supplemental Movies 4 and 5 to confirm location of the Cy3 TSA-positive material completely within the macrophage. Images were taken using a $\times 60$ Plan Apo Oil immersion NA1.40 (Olympus).

The pharmacological agents used in these studies were chosen because of their ability to delay apoptosis of human neutrophils *in vitro*. The actions of these groups of agents on neutrophil apoptosis are unrelated in the mechanism. It therefore seems unlikely that they are all having their effects via processes other than apoptosis. Moreover, although rates of detectable neutrophil apoptosis are low, agents that suppress apoptosis of neutrophils *in vitro*, including caspase inhibitors and inflammatory regulators such as LPS, also suppress them in this *in vivo* model.

The most potent inhibitor of inflammation resolution tested was zVD.fmk [9]. By comparison, the effects of other caspase inhibitors were relatively weak and only readily apparent when resolution was considered within individual fish. This may simply reflect the larger size and hence, poorer tissue penetration of the tetrapeptide inhibitors. Alternatively, the main target of zVD.fmk may be a caspase with different substrate specificities, either a different member of the caspase family or one of the caspases tested, but with different substrate preferences in zebrafish. However, there appears to be a high degree of homology in substrate specificities between mammalian and zebrafish caspases [30]. The doses of caspase inhibitors used in this study are high, and this will reduce the specificity of the caspase inhibitors used; thus, care should be taken when interpreting these results. However, the doses used are similar to those used in many experiments on human neutrophils (reviewed in ref. [31]).

The phenazine pigment pyocyanin, produced by *P. aeruginosa*, has been shown to induce apoptosis of neutrophils purified from human peripheral blood [20] and in the lungs of mice with experimental *Pseudomonas pneumonia* [21]. We now show that it is also able to reduce numbers of neutrophils during zebrafish-tailfin injury-induced inflammation in a caspase-dependent manner, further highlighting the similarities between this model and mammalian models of neutrophilic inflammation. Similarly, roscovitine is able to accelerate inflammation resolution in this model, as it is in mammalian models [2]. These compounds were used as positive controls to establish a compound-screening paradigm to assess this model for its role as a vehicle for anti-inflammatory drug discovery. Of the 12 compounds identified, six have known anti-inflammatory activity and include the corticosteroid flumethasone. Perhaps surprisingly in this context, corticosteroid treatment has been shown to delay the apoptosis of isolated human neutrophils [32, 33]. However, in an *in vivo* setting, it may well be that effects on neutrophil apoptosis are balanced by effects on other cell types, for example, reduction in inflammatory mediators by tissue cells adjacent to the site of injury.

Following an inflammatory insult, neutrophils move through tissues, up local gradients of chemotactic factors to provide defense against pathogenic organisms, and take up tissue debris. Tissue neutrophils subsequently meet one of three possible fates: they may be lost from the tissues into inflammatory exudates (e.g., into the airways [34], onto the dermis [35], or into other compartments, such as the pleura [36] or synovial space [37]); they may migrate away from the site of inflammation [24, 38–41]; or they may undergo apoptosis *in situ* and be taken up by macrophages, which subsequently undergo re-

verse chemotaxis [42]. Failure of these processes may lead to persisting inflammation and tissue damage. These disposal routes are not mutually exclusive, and the balance of the three routes may differ in different tissues in response to different stimuli and at different times. Of the three processes, *in vivo* evidence that neutrophil apoptosis is functional in the resolution of tissue neutrophilia has been hardest to obtain. Evidence for the *in vivo* importance of neutrophil apoptosis comes from visualization of apoptotic neutrophils in tissue exudates or in fixed tissue samples from a range of experimental and clinical scenarios. For example, apoptotic neutrophils have been demonstrated in lung lavage from patients with acute respiratory distress syndrome [28], in joint fluid of patients with rheumatoid arthritis [26, 37], and in pleural fluid in experimentally induced pleurisy [43]. In addition, it is possible to identify apoptotic neutrophils by histological examination of fixed samples taken from resolving inflammation *in vivo* [44]. Manipulation of neutrophil apoptosis has been shown to modulate inflammation resolution in experimentally induced pleurisy [2, 43], in bleomycin-induced lung injury, and serum-induced arthritis [2]. In all of these models, numbers of identifiable apoptotic neutrophils are low but are functionally important. The ability to visualize apoptosis of neutrophils as it occurs using tissue-specific apoptosis reporter transgenics and to observe the effects of pharmacological and genetic manipulations on neutrophil apoptosis in living tissues would be important additions to this field.

The data presented here show that neutrophil apoptosis contributes to the spontaneous resolution of inflammation in a zebrafish model of neutrophilic inflammation. Morphological and biochemical features of neutrophil apoptosis have been described, along with evidence of the functional consequence of neutrophil apoptosis *in vivo* (macrophage uptake). Moreover, targeted pharmacological modulation of neutrophil apoptosis leads to changes in amount of spontaneous inflammation resolution. This is in keeping with data demonstrating apoptotic neutrophils at sites of resolving inflammation in animal models of disease and human disease states [26, 28, 37]. The numbers of apoptotic neutrophils are low in this study but consistent with the percentage of apoptotic cells seen in other models [10, 27, 28]. These low levels of apoptosis can be suppressed further by caspase inhibitors, LPS, and dbcAMP, demonstrating that modulation of neutrophil apoptosis may explain, at least part, the action of these compounds on inflammation resolution. A unique aspect of this model is the ability to visualize the cellular events comprising the apoptotic program, as they occur in tissue neutrophils participating in the inflammatory response. Other models permit the analysis of neutrophils removed from inflammatory sites (albeit usually from exudates rather than from the tissues *per se*) or the post-fixation analysis of apoptosis. However, the direct visualization of the apoptotic program *in vivo* would appear to be a distinct possibility in this model, potentially giving a unique window into the resolution of neutrophilic inflammation.

The difficulty in identifying individual apoptotic neutrophils is compounded by the observed loss of GFP fluorescence as neutrophils undergo apoptosis. This loss of fluorescence was unexpected and may be cell type-specific. We have not ob-

served this phenomenon in a range of other tissue-culture cell types, in which GFP derivatives have been used to aid assessment of apoptosis [45, 46]. However, the loss of fluorescence in this context might explain the difficulty in identification of apoptotic neutrophils by other groups who have studied GFP neutrophils in vivo. The mechanism for the loss of fluorescence is not clear, although potential explanations include the quenching of fluorescence by hypochlorous acid [47] or digestion of GFP by granule proteases within the neutrophil. One explanation for the differences in number of TUNEL-positive neutrophils, when identified by anti-GFP antibody compared with histochemical staining, is loss of the GFP epitope during apoptosis. Definitive experiments to prove this will require generation of larvae deficient in mpx and granule proteases.

Work by other groups [24] has identified retrograde chemotaxis as a mechanism by which zebrafish inflammation may resolve, and there is evidence that a similar process occurs in certain human disease states [38]. Retrograde chemotaxis may coexist with neutrophil apoptosis as a mode of resolution of inflammation, and there may be circumstances in which one form of clearance may predominate. The exact balance of these two processes for each inflammatory scenario remains to be determined, and a complete analysis would also require quantitation of ongoing neutrophil recruitment. It seems likely that the balance of new neutrophil recruitment, retrograde chemotaxis, and clearance by apoptosis will be different for different inflammatory stimuli (including differing wound sizes and the presence or absence of pathogens), even within the zebrafish tailfin wound model. This would also be consistent with the observation that neutrophil apoptosis has not been observed in smaller wounds in comparison with the more extensive wounds used in these studies [24, 40]. The loss of GFP fluorescence during neutrophil apoptosis would mean that specific dual staining would be required, and observation alone would be insufficient. In addition, the use of older zebrafish (7–10 dpf) was necessary for us to identify conclusively apoptotic neutrophils, likely to be as a consequence of the increased numbers of neutrophils required to detect this transiently detectable in vivo event. In contrast, the numbers of neutrophils recruited to the smaller wounds of Day 3 or 4 larvae in other studies would be unlikely to result in detectable numbers of apoptotic cells. For example, Mathias et al. [24] and Brown et al. [40] show peak numbers of neutrophils of <10/fish, compared with the figure of almost 70/fish in these experiments. With rates of detectable apoptosis of 4.4%, they would be unlikely to be able to detect apoptotic cells, even had they stained specifically for them. The specificity of this particular transgenic zebrafish line makes it an ideal model for the future studies to determine the relative contributions of apoptosis and retrograde chemotaxis in the resolution of neutrophilic inflammation.

This study confirms neutrophil apoptosis occurs in tissues during inflammation resolution and is amenable to pharmacological manipulation. The unique features of zebrafish make them a powerful tool to study the in vivo fate of inflammatory cells following genetic or pharmacological perturbation of the apoptosis pathways. The advantages of this model will be exploited in forward genetic and “chemical genetic” screens to

gain an understanding of the controls of neutrophil apoptosis and identify compounds with the potential to hasten resolution of tissue neutrophilia in inflammatory disease.

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